Biocompatibility of PCL-Graphene Nanostructured Scaffolds with Mouse Embryonic Stem Cell-derived Cardiomyocytes

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Introduction

Since adult cardiomyocytes are not readily available for clinical use, numerous efforts have been made to derive functional cardiomyocytes from pluripotent stem cells. [1,2]. A variety of cardiovascular tissue engineering strategies have been explored to develop engineered cardiac tissues for *in vitro and in vivo applications utilizing fibrous tissue* scaffolds, both single polymer scaffolds and hybrids of polymers with hydrogels, coatings or embedded materials[3-9]. While graphene, a single layer carbon crystal, has recently become a material of interest for tissue engineering applications including osteogenic, neural and stem cell differentiation [10-12], its potential for cardiac tissue engineering is yet unknown. The inherent electro-activity of the myocardium makes graphene an especially attractive option for cardiac tissue engineering due to its high electrical conductivity. Thus, a novel hybrid 3D scaffold with graphene has been developed and its effect on the function of stem cell derived cardiomyocytes is examined.

Materials and Method

Scaffold development and characterization

• Scaffold Preparation: 15% polycaprolatone (PCL) and 0.01 wt% graphene/15 wt% PCL(PCL+G) scaffolds were prepared by electrospinning.

• Scaffold Characterization: Representative SEM images of 10% PCL and 10% PCL+G scaffold samples were obtained. Mechanical testing was also performed on 15%PCL and 15%PCL+G scaffold samples using an Instron at a rate of 10mm/min.

Mouse embryonic stem (mES) cell culture and differentiation

• mES (stable cardiac troponin T-eGFP line, generous gift from Dr. Yibing Qyang) was used. Cells were cultured on a fibroblast feeder layer for 2 days in DMEM medium supplemented with leukemia inhibiting factor (LIF) and additional 2 days without the feeder layer.

• Embryoid body (EB) were formed using a hanging drop method as previously described[13]

• EBs were cultured in IMDM differentiation medium supplemented with 5mg/mL ascorbic acid for 8 days and yielded spontaneously contracting cardiomyocytes

Seeding of scaffolds with mES cell derived cardiomyocytes (mES-CM)

• Spontaneously contracting CMs were digested using a collagenase A and B solution and purified using a discontinuous Percoll separation method.

• Purified mES-CMs were then seeded at 8x106 cells/cm2 on PCL or PCL+G scaffold and cultured for 6 days.

• Live/Dead assay was performed to determine viability of mES-CMs on scaffolds.

Characterization of mES-CM (2D and 3D)

• Beating frequency: The rate of spontaneous contraction of mES-CM after 6 days in culture was measured.

• Immunofluorescence Imaging: Immunofluorescent images were acquired from GFP positive mES-CM expressing cTNT while in culture. To visualize F-actin, fixed mES-CM in 2D and 3D culture were stained with rhodamine-conjugated phalloidin for 2hrs at room temperature and imaged using a confocal fluorescence microscope (Olympus).

• Protein Analysis: Cell lysates of mES-CM in 2D and 3D cultures were prepared in a RIPA buffer solution with 1% Triton-X-100. Blots were probed for cardiac troponin T (cTnT), myosin heavy chain (MHC) and connexin43 (Cx43). Fresh fetal rat heart tissue homogenates and mES cell lysates acted as controls. Beta actin was used as a loading
control.

• Calcium Handling: Intracellular calcium was measured via loading with Rhod-2AM and 0.016% (wt/wt) pluronic for 30 minutes. Fluorescence was recorded using an Andor Ixon CCD camera at 50 frames/s and analyzed using INDEC Imaging Workshop. Measurements were obtained from spontaneous beats as well as after exposure to10mM
caffeine and 1um isoproterenol.

Results

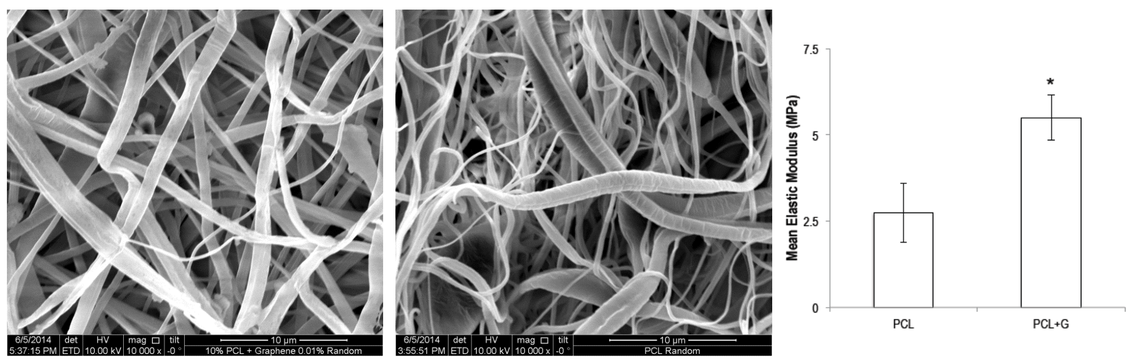
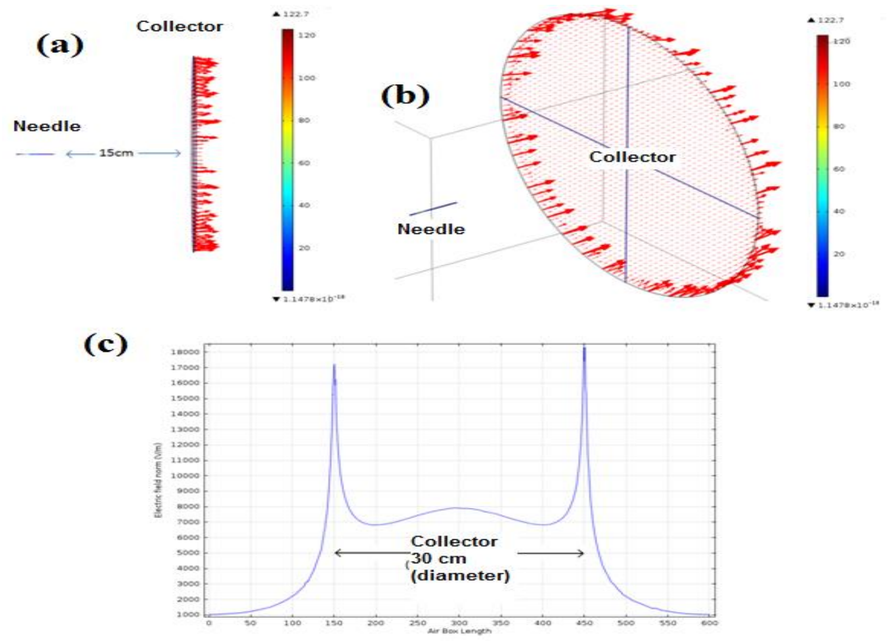
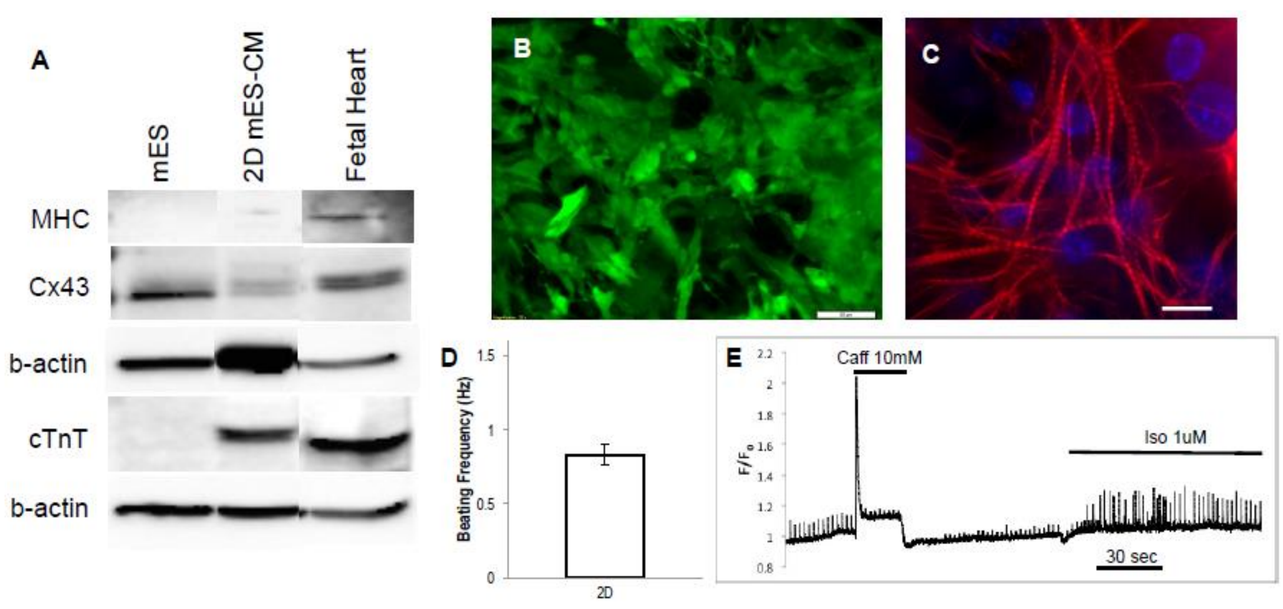


Fig. 1. Representative SEM images of (A) 10% PCL and (B) 10% PCL+G scaffolds. Scale bar=10um. (C) Mean elastic modulus of 15% PCL and 15%PCL+G scaffolds are 2.75±0.85 MPa and 5.50±0.64 MPa. (n=4 ± SEM)



Electric Field modeling and analysis showing the electric field pattern at the collector surface. (a) Distance between the needle (positive terminal) and the collector (ground) showing the electric field lines. The length and thickness of the arrows is proportional to the intensity of the field (maximum of 122.7 V/m), (b) a 3-D view of the model showing the electric field predominantly over the periphery of the disc collector anticipating a larger accumulation of nanofibers over the periphery, (c) line graph plot showing the highest intensity of the electric field at the periphery of the 30cm diameter collector. The difference in field strength (~1000 V/m) between the two edges is due to the error in positioning the arc across the disc collector.



(A) Western analysis of mES-CM revealed expression of cardiac specific markers such as MHC, Cx43 and cTnT. **(B) mES-CMs highly express cTnT-eGFP as shown in green, scale bar=100um. (C) F-actin staining** of mES-CMs with rhodamin-conjugated phalloidin (red) shows well-registered sarcomeres, scale bar=10um. **(D) Average spontaneous beating frequency of mES-CM is approximately 0.83±0.07 Hz. (n=11±SEM) (E)** mES-CM beat spontaneously and respond to caffeine (Caff), by releasing all stored calcium from the sarcoplasmic reticulum resulting in a large transient seen on the trace. Isoproterenol (Iso) resulted in a higher frequency of transients as shown on the trace.

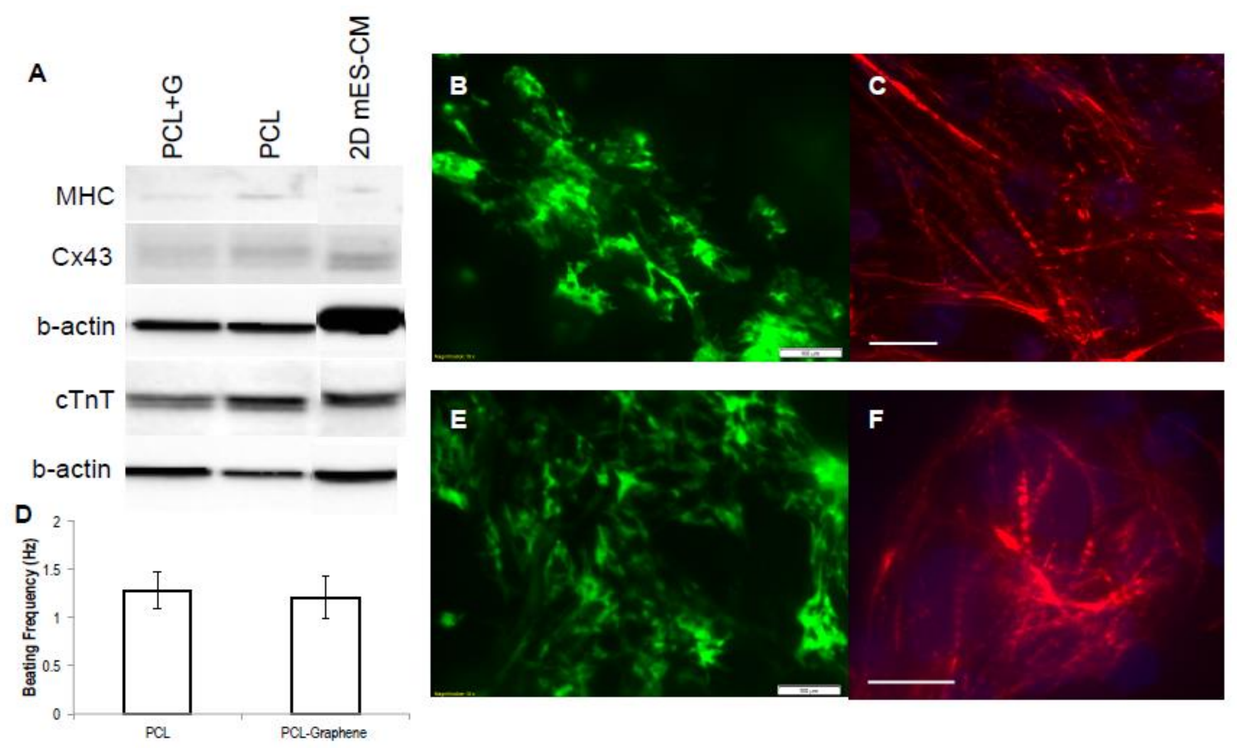


Fig. 3. (A) Western blot analysis of mES-CM seeded on PCL and PCL+G scaffolds demonstrates expression of classical cardiac markers such as MHC, Cx43 and cTnT. **(B,E) Spontaneously contracting mES-CM on** PCL and PCL+G scaffolds highly express cTnT-eGFP, scale bar=100um. **(C,F) F-actin staining with rhodaminconjugated** phalloidin (red) shows well-registered sarcomeres on both PCL and PCL+G scaffolds, scale bar=10um. **(D) Average spontaneous beating frequency of mES-CM on PCL and PCL+G scaffolds is** approximately 1.28±0.20 Hz (n=7±SEM) and 1.21±0.22 Hz (n=8±SEM) respectively.

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